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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
During development, neurons become dependent on target-derived neurotrophins for survival and maintenance of differentiated functions. Failed or inappropriate target interactions in vivo, or withdrawal of neurotrophins in vitro, lead to a characteristic sequence of molecular cell death events termed "apoptosis". The purpose of the proposed research is to examine the roles of the *Nf1* gene product, neurofibromin, in modulating the apoptotic response to neurotrophin withdrawal, as well as the survival response to depolarization. Previously, we demonstrated that many sensory neurons isolated from *Nf1*-/- mouse embryos survive in the absence of neurotrophins. The scope of the experiments for Year 2 included: 1) assessing synergy between the survival-promoting effects of neurotrophins and activity-mediated signaling, 2) characterizing the apoptotic response to ceramide, 3) identifying signaling components that mediate depolarization rescue, and 4) incorporating sympathetic neurons into the analyses of Nf1-deficiency and responses to neurotrophin withdrawal. Our results indicate that Nf1-/- sensory and sympathetic neurons differ in their resistance to apoptosis initiated by NGF withdrawal, and propose that the timing of target contact may in part account for these differences. In addition, we have confirmed that Nf1 haploinsufficiency increases resistance to apoptosis and sensitivity to rescue by depolarization for both sensory and sympathetic neurons.

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INTRODUCTION

During development, peripheral neurons become dependent on target-derived neurotrophins for survival and maintenance of differentiated functions. Failed or inappropriate target interactions in vivo, or withdrawal of neurotrophins in vitro, lead to a characteristic sequence of molecular cell death events termed "apoptosis". The purpose of the proposed research is to examine the roles of the *Nf1* gene product, neurofibromin, in modulating the apoptotic response to neurotrophin withdrawal, as well as the survival response to depolarization. We have shown that many sensory, and almost all sympathetic neurons isolated from *Nf1*-/- mouse embryos survive in the absence of neurotrophins (Vogel et al., 1995). Based on the advice of the reviewer of last year's progress report, we have begun to analyze the responses of E13, E15, and E17 SCG sympathetic neurons, isolated from Nf1+/+, +/-, and -/- embryos, to NGF withdrawal, activity-mediated survival signaling, and suboptimal neurotrophin dosages.

BODY

Task 1. Characterize responses of *Nf1*+/+ and *Nf1*+/- DRG and retinal neurons to activity-mediated survival signaling *in vitro*. (Months 0-6)

DRG Neurons. During the first year of funding, we characterized survival responses of embryonic mouse dorsal root ganglion (DRG) sensory neurons to different concentrations of depolarizing KCl, following nerve growth factor (NGF) withdrawal at 24, 48, or 72 hours *in vitro*. Levels of intracellular calcium and potassium are known to influence apoptosis, particularly in postmitotic neurons (reviewed by Yu et al., 2001). Our hypothesis was that neurons with lower amounts of neurofibromin (*Nf1*+/-) would be more sensitive to the survival-promoting effects of high potassium than would wild-type (*Nf1*+/+) neurons, due to higher levels of activated Ras. In last year's progress report, we demonstrated significant, reproducible differences in both apoptosis rate and survival response to depolarizing levels of potassium, between DRG neurons isolated from *Nf1*+/+ and *Nf1*+/- mouse embryos at E15 and E17.

With our standard paradigm of NGF withdrawal at 72 hours *in vitro*, we found no statistically significant differences between the responses of *Nf1*+/+ and *Nf1*+/- DRG neurons isolated at E13. To reduce neurofibromin levels further, without eliminating the protein completely, we crossed *exon23a*-/- mice with *Nf1*+/- mice, to obtain litters of embryos with genotypes *exon23a*-/*Nf1*+ and *exon23a*-/*Nf1*-. Again, we found no differences in E13 DRG neuron rescue by depolarizing KCl; however, by E15, this additional reduction in neurofibromin had a significant effect on the survival response of sensory neurons (data not shown).

SCG Sympathetic Neurons. As described in last year's report, we experienced difficulties with the retinal neuron cultures, and it was recommended that we use sympathetic neurons, isolated from the superior cervical ganglion (SCG). This turned out to be a fortuitous choice, since when we performed the NGF withdrawal/KCl rescue experiments with E15 SCG populations, the differences between the responses of *Nf1*+/+ and *Nf1*+/- neurons were even more marked than were the differences for DRG sensory populations (**Figure 1**). Again, at E13, we have so far observed no significant difference between *Nf1*+/+ and *Nf1*+/- SCG neuron responses. We are currently completing this series of experiments with E13 and E17 SCG neurons, and now propose to combine these data with those obtained for Task 3 (see below; both DRG and SCG neurons) in a manuscript to be submitted to *J. Neuroscience* this fall.

Task 2. Identify signal transduction pathways activated by KCl-mediated depolarization in DRG and retinal neurons. (Months 6-12)
Nikita Ruparel, a rotating graduate student, and I used standard inhibitors of PI3 kinase and MAP kinase, in our NGF withdrawal paradigm for Nf1-/-, +/-, and +/+ DRG neurons. To date, our results are consistent with those previously reported, in that survival signaling appears to be mediated primarily by the PI3 kinase pathway. Since we have optimized the NGF withdrawal and KCl rescue conditions, we expect to complete these experiments within the next 4-6 months without complications. At the same time, we will include nifedipine, which blocks L-type calcium channels, in our KCl depolarization studies. These signal transduction data would complement the results obtained in Tasks 1 and 3, and could be included in the manuscript; they confirm published reports, and are not sufficiently novel to stand on their own.

Task 3. Characterize synergy of neurotrophin- and activity-mediated survival signaling in *Nf1*+/+ and *Nf1*+/- DRG and retinal neurons. (Months 12-18)
DRG Neurons. To date, we have focused on the survival responses of E17/E18 DRG neurons to depolarizing KCl and suboptimal NGF concentrations, following NGF withdrawal at 72-96 hours *in vitro*. At this stage, DRG neurons are less sensitive to NGF withdrawal, but significant differences between *Nf1*+/+ and *Nf1*+/- cells are apparent (**Figure 2**). The increased sensitivity of *Nf1*+/- neurons to low doses of NGF is consistent with our previous data utilizing *exon23a*-/- DRG, trigeminal, and SCG neurons (Brannan and Vogel, in preparation). We are completing these experiments with E15 *Nf1*+/+ and +/- DRG neurons.
SCG Neurons. **Figure 1** shows that E15 SCG sympathetic neurons isolated from *Nf1*+/- embryos are more sensitive to depolarizing KCl and to low doses of NGF, when compared to neurons isolated from *Nf1*+/+ littermates. However, it is difficult to say at this point whether there is synergy between neurotrophin and activity-mediated signaling, or if there is simply an additive effect. We are completing these experiments with more data from E15 litters, and with neurons isolated from E17 mouse embryos, and propose to combine the data from Tasks 1 and 3 for a full-length manuscript, to be submitted to *Journal of Neuroscience*.

Task 4. Characterize the role of neurofibromin in mediating neuronal apoptosis following neurotrophin withdrawal. (Months 0-6)
DRG Neurons. During the first year of funding, we characterized the response of E12.5 *Nf1*-/- DRG neurons to NGF withdrawal and KCl rescue. Based on our previous results (Vogel et al., 1995), we predicted that *Nf1*-/- DRG neurons would not undergo apoptosis following NGF withdrawal, whereas neurons isolated from *Nf1*+/+ and +/- littermates should die 24-48 hours after NGF is removed. To our surprise, we found that approximately 50% of E12.5, and 60% of E13.0, *Nf1* mutant DRG neurons undergo apoptosis within 24-48 hours of NGF withdrawal (**Figure 3**); however, this percentage does not increase significantly over the subsequent 3-4 days in culture (data not shown). In contrast to the loss of many *Nf1*-/- DRG neurons following NGF removal, we did not observe apoptosis in sister cultures of neurofibromin-deficient neurons that had never been exposed to NGF *in vitro* (data not shown). For E13 *Nf1*-/- DRG neurons deprived of NGF, depolarizing KCl effects a complete rescue, and a suboptimal dose of NGF (0.5ng/ml) rescues over 80% of the cells (**Figure 3**). In contrast, fewer than 40% of E13 *Nf1*+/- DRG neurons can be rescued by activity-mediated signaling, and over 90% undergo apoptosis in the presence of the low dose of NGF (**Figure 3**).

Based on the above, and other unpublished results, we propose that target contact, and concomitant exposure to neurotrophins, initiates the development of neurotrophin dependence in peripheral neurons, even if they lack neurofibromin. Environmental cues encountered by growing axons *en route* to the target undoubtedly influence acquisition of neurotrophin dependence; in cultures of ganglia isolated from developing embryos, some neurons may have extended axons towards or even contacted the peripheral target, whereas others have not yet developed axons.

SCG Neurons. We reported previously that SCG neurons isolated from *Nf1*-/- embryos never develop neurotrophin dependence *in vitro*, regardless of NGF exposure and withdrawal paradigms (Vogel et al., 1995). This may reflect the fact that few, if any, SCG neurons isolated at E13.5 (the latest stage to which *Nf1*-/- mouse embryos survive) have extended axons towards peripheral targets, and certainly none have contacted their targets *in vivo*. To determine whether NGF dependence could be induced in sympathetic neurons by exposure to the neurotrophin, we subjected E12.5 *Nf1*-/- and +/- SCG neurons to the NGF withdrawal paradigm described above for DRG neurons. **Figure 4** shows that NGF dependence fails to develop in 60% of *Nf1*+/- SCG neurons isolated at this early stage, and that very few of these cells can be rescued by depolarization. In contrast, NGF withdrawal has very little effect on the survival of SCG neurons isolated from *Nf1*-/- littermates (**Figure 4**). *These results raise the possibility that the role of neurofibromin in regulating acquisition of neurotrophin dependence may be very different for sensory and sympathetic neurons.* We propose to present our results, comparing NGF withdrawal responses of Nf1-/- DRG and SCG neurons, in a manuscript to be submitted to *J. Neuroscience* or *J. Neurobiology*. This manuscript will include the results of experiments involving: pre-target contact DRG neurons (E11.5), "pulses" of NGF exposure (DRG and SCG), survival responses to suboptimal NGF doses (DRG and SCG), and prolonged (7-10 days) NGF exposure (SCG). In addition, we may include immunocytochemical analyses of c-jun kinase and c-jun phosphorylation in response to NGF withdrawal, for E13 Nf1-/- and Nf1+/- sensory and sympathetic neurons (see Task 7).

Task 7. Characterize the susceptibility of neurofibromin-deficient neurons to ceramide-mediated apoptosis. (Months 12-30)

In cultured cortical neurons, increased ceramide levels, generated by cleavage of sphingomyelin, activate c-jun and p38 kinases following the induction of apoptosis (Williame-Morawek et al., 2003). Addition of cell-permeable forms of ceramide induces apoptosis in cultured neurons and neuronal cell lines, and this apoptotic response can be blocked by overexpression of PI3 kinase or constitutively activated Akt (Zhou et al., 1998; Goswami et al., 1999). We proposed that Nf1-/- sensory neurons might be resistant to ceramide-induced apoptosis, due to constitutive activation of the PI3 kinase/Akt survival signaling pathway. **Figure 5** shows the results of experiments in which a cell-permeable C2-ceramide was added to cultures of E12.5 *Nf1*-/- and +/- DRG neurons at different concentrations. In contrast to our predictions, ceramide can induce apoptosis in neurofibromin-deficient neurons, even in the presence of NGF (**Figure 5**). We are currently repeating these experiments and following apoptosis at 6-hour intervals; in addition, we plan to assess the effects of C2 -ceramide on E13 *Nf1*-/- and +/- SCG sympathetic neurons. In a recent paper on ceramide-induced apoptosis in cultured cortical neurons, both c-jun kinase (JNK) and c-jun phosphorylation were examined immunocytochemically (Williame-Morawek et al., 2003). We propose to examine the time course of phosphorylation of these proteins in E13 *Nf1*-/- and +/- DRG and SCG neurons, following C2-ceramide exposure.

KEY RESEARCH ACCOMPLISHMENTS

- Performed NGF withdrawal and KCl rescue experiments for E13, E15, and E17 SCG sympathetic neurons, isolated from Nf1+/+ and +/- mouse embryos, to complement previous results with DRG sensory neurons (Task 1).
- Examined possible "synergy" between neurotrophin- and activity-mediated survival signaling for Nf1+/- and +/+ DRG neurons; began similar experiments with SCG neurons (Task 3).
- Characterized effects of NGF withdrawal on E12.5 *Nf1*-/- SCG sympathetic neurons, to complement previous results with DRG sensory neurons (Task 4).
- Characterized apoptotic effects of C2-ceramide on E12.5/E13 Nf1-/- and +/- DRG neurons.

REPORTABLE OUTCOMES

Manuscripts in preparation:

*Brannan, C.I., and Vogel, K.S. (in preparation) Increased neurotrophin sensitivity and delayed apoptosis in sensory and sympathetic neurons that lack an alternatively spliced form of neurofibromin. *To be submitted to the Journal of Neuroscience.*

*Vogel, K.S. (in preparation) Nf1 haploinsufficiency alters neurotrophin sensitivity and the response to NGF withdrawal in developing sensory and sympathetic neurons. *To be submitted to the Journal of Neuroscience.*

Vogel, K.S. (in preparation) Differential responses of neurofibromin-deficient sensory and sympathetic neurons to neurotrophin exposure and withdrawal.

* These two papers address the effects of reduction of neurofibromin levels on responses to neurotrophin- and activity-mediated survival signaling in sensory and sympathetic neurons, using different gene targeting approaches in mice. Therefore, we plan to submit the manuscripts for possible co-publication.

Funding obtained

Dates: Sept. 1 2004- Aug. 31 2005. Title: "Nf1 and Mrg15 Regulation of Tumorigenesis and Neural Cell Proliferation" Amount: $15,000 Source: San Antonio Cancer Institute/NCI. The role of Nf1 in regulating cortical precursor and sympathetic neuroblast proliferation will be examined, in the context of possible interaction with cell cycle-regulating gene Mrg15.

Employment and Training Opportunities:

Robert Hudson was hired as a Senior Research Assistant in November 2003. Robert performs genotyping on all experiments for the project, so that the neuronal cultures are counted "blind" to genotype by the principal investigator. Robert also assists with preparation of culture dishes, NGF withdrawal experiments, and immunocytochemistry

Nikita Ruparel is a graduate student in the Department of Cellular and Structural Biology, and performed experiments with PI3 kinase and MAP kinase inhibitors. During her rotation in the PI's laboratory, she learned embryo microdissection and neuronal cell culture techniques.

CONCLUSIONS

Importance and Implications. Our results to date support the emerging idea that neurofibromin expression and *Nf1* haploinsufficiency influence the behavior of both peripheral and central neurons. Loss of neurofibromin, with the resulting abnormalities in Ras and PI3 kinase signaling, has profound effects on the neurotrophin dependence and sensitivity of embryonic sensory neurons (Vogel et al., 1995; Klesse and Parada, 1998; Vogel et al., 2000). Behavioral experiments with *Nf1*+/- and *exon23a*-/- mice indicate that neurofibromin function in CNS neurons modulates learning and memory (Silva et al., 1997; Costa et al., 2001). Over the past year, we have incorporated the reviewer's suggestion to utilize sympathetic neurons (from the superior cervical ganglion, SCG) in the experiments outlined in Tasks 1, 3, and 4. We have shown that *Nf1* haploinsufficiency affects both neurotrophin- and activity-mediated survival signaling for sensory and sympathetic neurons, at least by embryonic day 15 in the mouse; the differences between *Nf1*+/- and +/+ neurons appear to become more significant with age. Our results may have implications for two areas: 1) the pathogenesis of learning disabilities in children with NF1, and 2) therapeutic strategies or targets for prolonging neuron survival, or for increasing neuronal response to protective agents, following injury or damage.

Neurotrophin withdrawal experiments involving E12.5 *Nf1*-/- and +/- SCG neurons revealed a difference between sympathetic and sensory neurons at this early stage. Whereas a proportion of neurofibromin-deficient DRG neurons die after NGF withdrawal (given prior NGF exposure), none of the NGF-deprived SCG neurons underwent apoptosis, even after a period of several days. Experiments with younger DRG neurons and with differential exposure to NGF, in addition to analyses of JNK and c-jun phosphorylation in SCG and DRG neurons, should begin to reveal the basis for these differences in the response of *Nf1*-/- neurons.

Changes to the Research Plan.

- We received the recommendation to use SCG sympathetic neurons, as a replacement for retinal neurons, in Spring 2004. Although this advice is excellent and will be useful in identifying differences in neurofibromin function for diverse neuronal types, we are running 6-8 months behind the proposed Statement of Work for many of our experiments. The SCG experiments for Tasks 1 and 3 are nearly complete, and when combined with the DRG results from last year should result in a substantial paper on *Nf1* haploinsufficiency in neurons. Comparison of SCG and DRG Nf1-/- neuron responses to NGF exposure and withdrawal has revealed intriguing differences, and we would like to include a few additional experiments that address mechanisms, to be included in a second manuscript.
- We did not expect that C2-ceramide would induce apoptosis in Nf1-/- DRG neurons (Task 7). Since Nf1-/- SCG neurons respond differently to NGF withdrawal, we propose to examine the effects of C2-ceramide on these sympathetic neurons as well. In addition, analyses of JNK and c-jun phosphorylation in this apoptosis paradigm will complement the experiments with c-jun-deficient mouse embryos for Year 3 (Task 8). In light of this, we may choose to focus more on the c-jun pathway, at the expense of experiments on the p75 NGFR pathway. If the funding period could be extended, without any changes to the actual budget, this would allow us to complete all of the proposed experiments, and

include the important comparisons between sensory (DRG) and sympathetic (SCG) neurons.

"So What" Section. The learning disabilities associated with NF1 constitute a highly variable phenotype, and in addition represent a controversial topic of research and clinical interpretations. Using mice that harbor targeted mutations in *Nf1*, Silva and colleagues have demonstrated that aberrant or reduced regulation of Ras signaling by neurofibromin may contribute to certain aspects of the spatial learning disorder (Silva et al., 1997; Costa et al., 2001). More recently, these researchers have proposed that the excessive Ras activity in *Nf1*+/- neurons leads to increased GABA-mediated inhibition and defects in long-term potentiation (Costa et al., 2002). Our results in Tasks 1 and 3, for both sensory and sympathetic neurons, are consistent with the interpretation that *Nf1*+/- neurons may respond aberrantly to electrochemical (ion gradients) and neurotrophin stimuli, which could potentially affect neuronal function and synaptic transmission. To relate these *in vitro* results to the complex issue of NF1-related learning disorders, it may be of interest to use computer modeling to characterize possible consequences of additional neurons (particularly inhibitory ones) in a circuit, or of aberrant signaling by neurons within a given circuit. Our results with *Nf1* haploinsufficient neurons also point to neurofibromin as a possible therapeutic target following neuronal injury; reduction in neurofibromin activity may prolong neuron survival, or enhance the response to protective agents.

Both neurotrophin signaling and activity-mediated processes are required to achieve correct target innervation patterns and synaptic plasticity in the peripheral nervous system (Davies, 2003). For many types of embryonic neurons, activation of the intracellular signaling pathways required for these processes often correlates with the timing of target contact. To date, our experiments indicate possible differences in the role of neurofibromin in acquisition of neurotrophin dependence, for embryonic sensory and sympathetic neurons. Both molecular mechanisms and the importance of target contact can be addressed readily in our *in vitro* system, and should contribute to our understanding of how neurotrophin signaling pathways are established during development.

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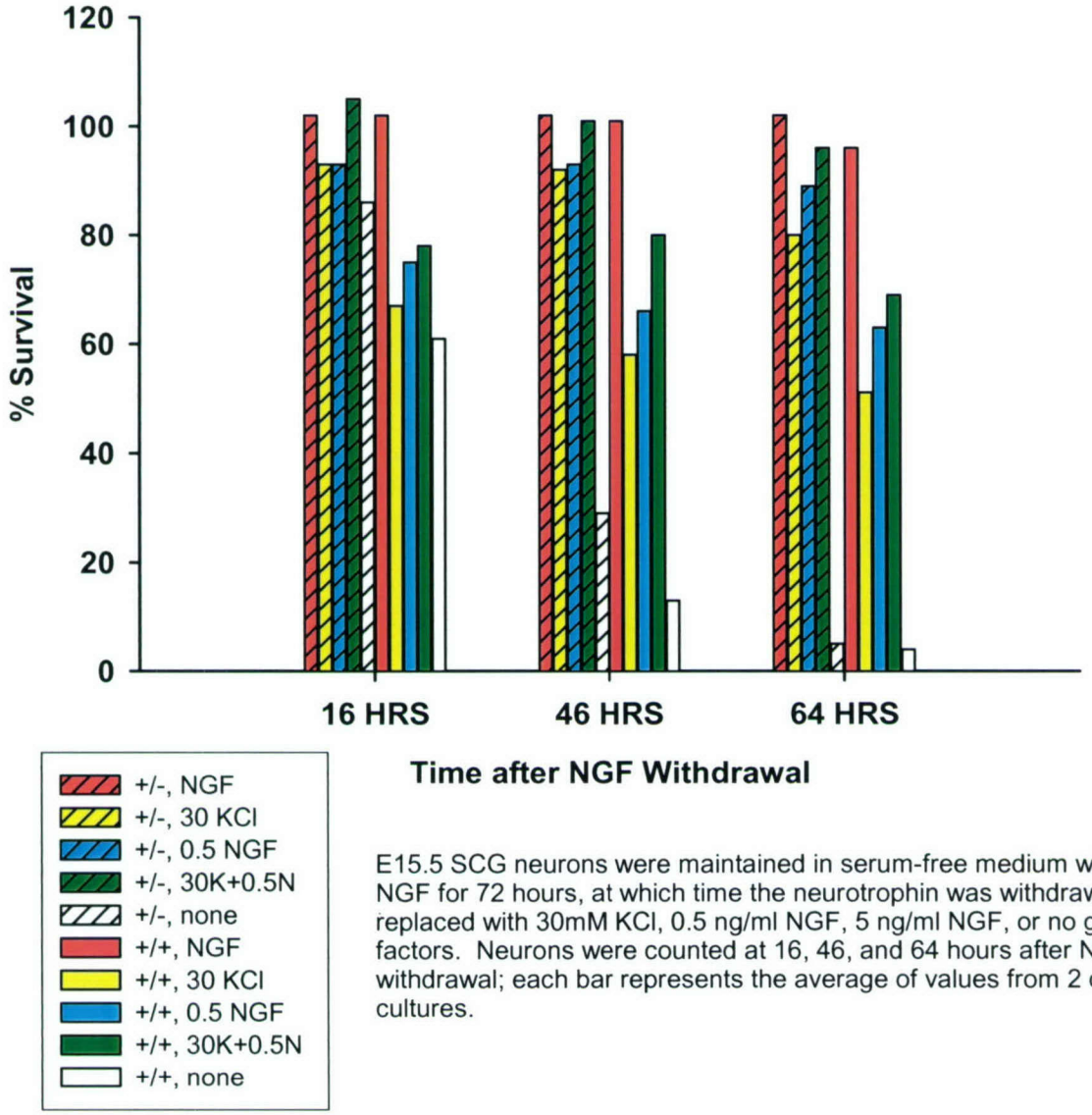
APPENDICES

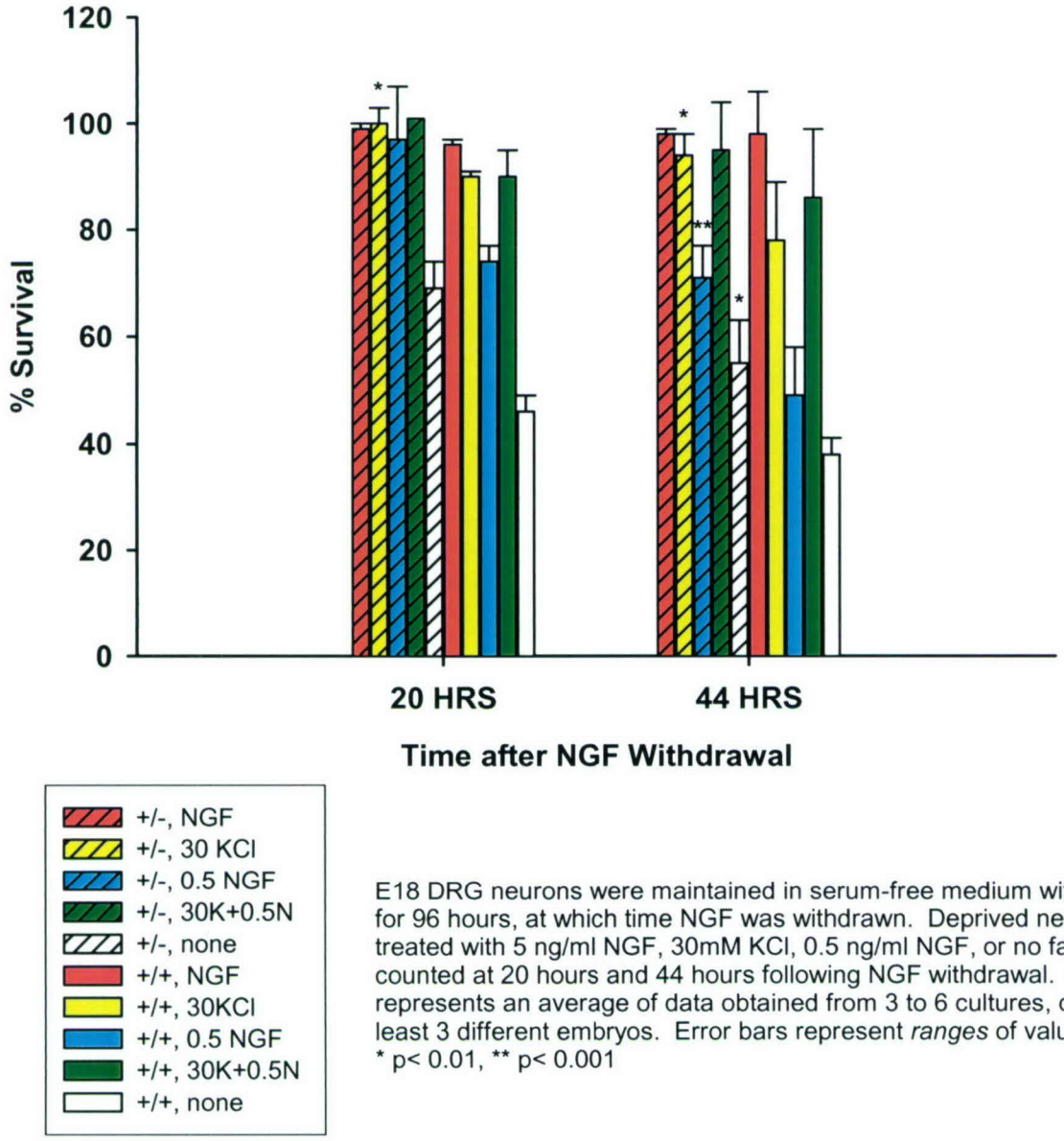
Figures 1-5

Figure 1.
Survival Following NGF Withdrawal at 72 Hours *In Vitro*
E15.5 SCG Sympathetic Neurons, Nf1+/- vs. Nf1+/+



E15.5 SCG neurons were maintained in serum-free medium with 5 ng/ml NGF for 72 hours, at which time the neurotrophin was withdrawn and replaced with 30mM KCl, 0.5 ng/ml NGF, 5 ng/ml NGF, or no growth factors. Neurons were counted at 16, 46, and 64 hours after NGF withdrawal; each bar represents the average of values from 2 or more cultures.

Figure 2.
Survival Following NGF Withdrawal at 96 Hours *In Vitro*
E18.0 DRG Sensory Neurons, Nf1+/- vs. Nf1+/+



E18 DRG neurons were maintained in serum-free medium with 5 ng/ml NGF for 96 hours, at which time NGF was withdrawn. Deprived neurons were treated with 5 ng/ml NGF, 30mM KCl, 0.5 ng/ml NGF, or no factors, and counted at 20 hours and 44 hours following NGF withdrawal. Each bar represents an average of data obtained from 3 to 6 cultures, derived from at least 3 different embryos. Error bars represent *ranges* of values obtained.
* $p < 0.01$, ** $p < 0.001$

Figure 3.
Survival Following NGF Withdrawal at 72 Hours *In Vitro*
E13.0 DRG Sensory Neurons, Nf1-/- vs. Nf1+/-

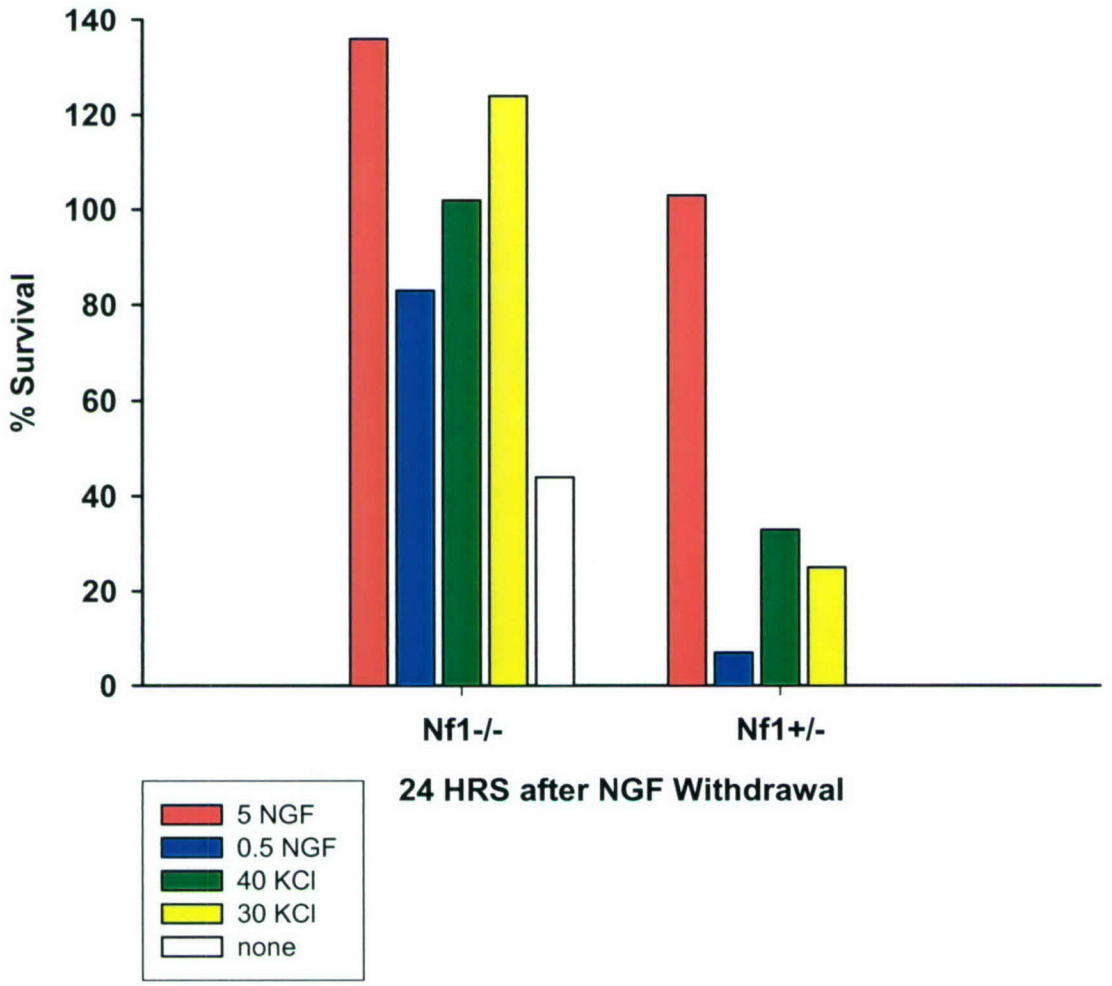


Figure 4.
Survival Following NGF Withdrawal at 72 Hours *In Vitro*
E12.5 SCG Sympathetic Neurons, Nf1-/- vs. Nf1+/-

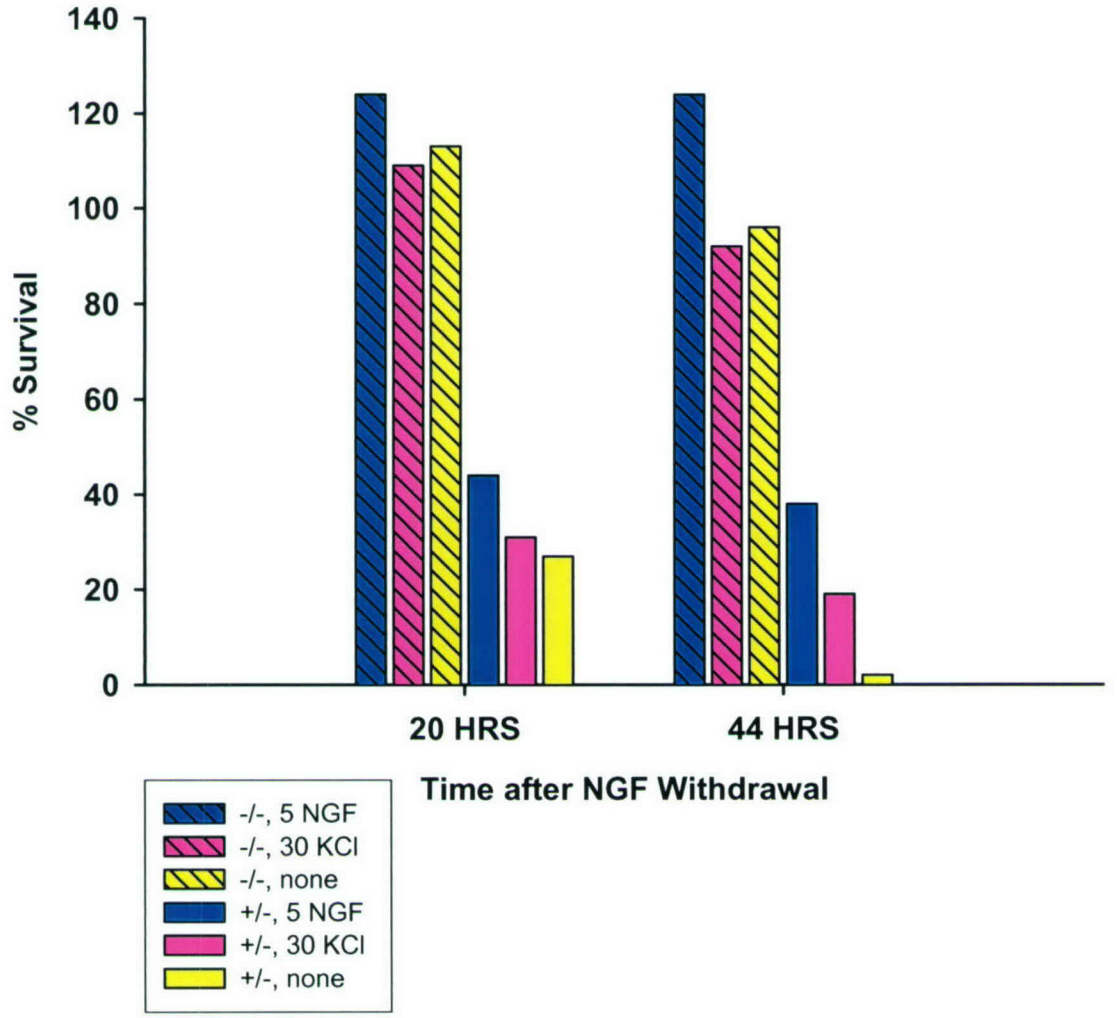
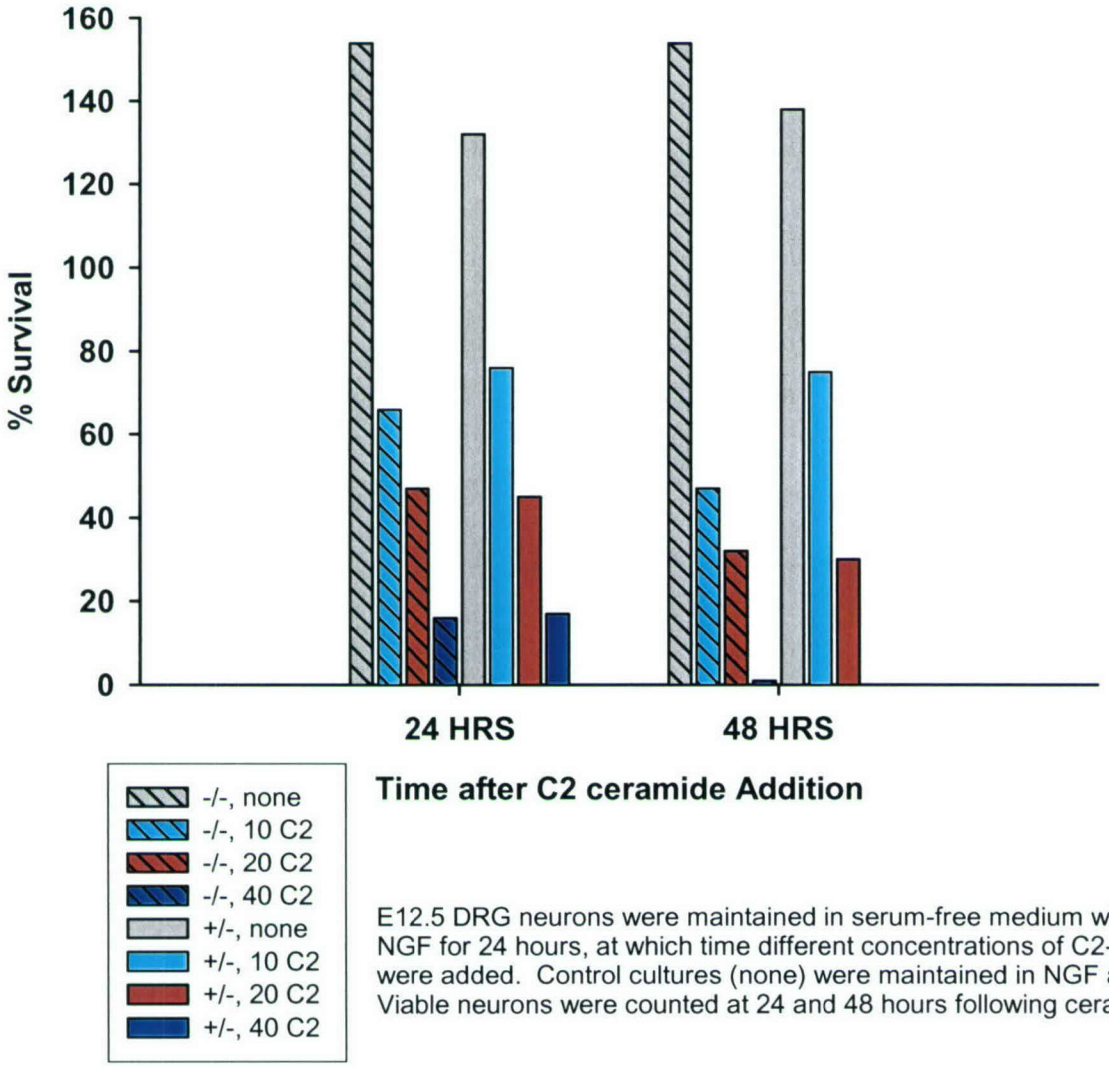


Figure 5.
Induction of Apoptosis by C2 Ceramide
E12.5 DRG Sensory Neurons, Nf1-/- vs. Nf1+/-



E12.5 DRG neurons were maintained in serum-free medium with 5 ng/ml NGF for 24 hours, at which time different concentrations of C2-ceramide were added. Control cultures (none) were maintained in NGF alone. Viable neurons were counted at 24 and 48 hours following ceramide addition.